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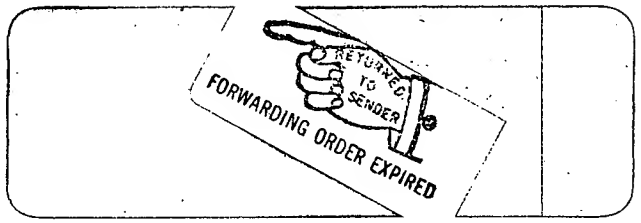

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/979,549	05/12/2003	Marie-Francoise Gautier	ORES10.001APC	5002

7590 09/12/2005

Knobbe Martens Olson & Bear
Sixteenth Floor
620 Newport Center Drive
Newport Beach, CA 92660-8016

EXAMINER	
COLLINS, CYNTHIA E	
ART UNIT	PAPER NUMBER
1638	

DATE MAILED: 09/12/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

NEW CENTRAL FAX NUMBER

Effective July 15, 2005

On July 15, 2005, the Central FAX Number will change to **571-273-8300**. This new Central FAX Number is the result of relocating the Central FAX server to the Office's Alexandria, Virginia campus.

Most facsimile-transmitted patent application related correspondence is required to be sent to the Central FAX Number. To give customers time to adjust to the new Central FAX Number, faxes sent to the old number (703-872-9306) will be routed to the new number until September 15, 2005. After September 15, 2005, the old number will no longer be in service and **571-273-8300** will be the only facsimile number recognized for "centralized delivery".

CENTRALIZED DELIVERY POLICY: For patent related correspondence, hand carry deliveries must be made to the Customer Service Window (now located at the Randolph Building, 401 Dulany Street, Alexandria, VA 22314), and facsimile transmissions must be sent to the Central FAX number, unless an exception applies. For example, if the examiner has rejected claims in a regular U.S. patent application, and the reply to the examiner's Office action is desired to be transmitted by facsimile rather than mailed, the reply must be sent to the Central FAX Number.

Office Action Summary	Application No.	Applicant(s)	
	09/979,549	GAUTIER ET AL.	
	Examiner	Art Unit	
	Cynthia Collins	1638	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 May 2003*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-7 and 9* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-7 and 9* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *19 November 2001* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☒ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *1101,0502*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Specification

The specification is objected to because it does not contain reference to and brief description of the drawing(s) as set forth in 37 CFR 1.74. See MPEP § 608.01(f).

Claim Rejections - 35 USC § 112 and 101

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-7 and 9 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The claims are drawn to a promoter comprising a nucleic acid fragment comprising at least one specific functional domain of the promoter of the TaTrxh2 gene, including a fragment wherein the sequence extends from position -1 to position -1111 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -1 to position -83 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -451 to position -591 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -591 to position -1111 relative to the ATG codon of the TaTrxh2gene; a fragment wherein the sequence extends from position -228 to position -451 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -451 to position -591

relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -83 to position -228 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence is that of the first intron of the TaTrxh2 gene. The claims are also drawn to an expression cassette, a recombinant vector, a transformed plant cell, a transgenic plant including a monocotyledonous plant, and a method for controlling the expression of a gene of interest in a plant cell.

The specification describes the TaTrxh2 gene as encoding a soft wheat (*Triticum aestivum*) thioredoxin h protein, the sequence of said gene and of the region in 5' comprising the promoter are represented in the attached sequence listing under the number SEQ ID NO: 1 (pages 2-3). The specification describes the 5' non-coding region (promoter) of TaTrxh2 gene as comprising the nucleic acid fragment which is represented in the attached sequence listing by the sequence SEQ ID NO: 2, and which is also represented in Figure 1, and which extends from position -1 to position -1111 relative to the ATG initiation codon of the TaTrxh2 (page 3). The specification describes specific structural components of the 5' non-coding region (promoter) of TaTrxh2 gene at pages 9-12, and the specification describes the functional characterization of the 5' non-coding region (promoter) of TaTrxh2 gene and four specific truncations (fragments) thereof at pages 12-20. The specification does not describe other sequences that are designated as "TaTrxh2", or as "TaTrxh2" promoter sequences.

The Federal Circuit has recently clarified the application of the written description requirement to nucleotide sequences. The court stated that "A description of a genus of cDNAs may be achieved by means of recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus." See *University*

of California v. Eli Lilly and Co., 119 F.3d 1559, 1569; 43 USPQ2d 1398, 1406 (Fed. Cir. 1997). The court has also stated that "a cDNA is not defined or described by the mere name "cDNA," even if accompanied by the name of the protein that it encodes, but requires a kind of specificity usually achieved by means of the recitation of the sequence of nucleotides that make up the cDNA. *See Fiers,* 984 F.2d at 1171, 25 USPQ2d at 1606.

In the instant case Applicant has not described a representative number of species falling within the scope of the claimed genus which encompasses promoter sequences designated "TaTrxh2", nor the structural features unique to the genus. Applicant also has not defined the type of promoter being claimed by its structure.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 9 provides for the use of the promoter of Claim 1, but, since the claim does not set forth any steps involved in the method/process, it is unclear what method/process applicant is intending to encompass. A claim is indefinite where it merely recites a use without any active, positive steps delimiting how this use is actually practiced.

Claim 9 is rejected under 35 U.S.C. 101 because the claimed recitation of a use, without setting forth any steps involved in the process, results in an improper definition of a process, i.e., results in a claim which is not a proper process claim under 35 U.S.C. 101. See for example *Ex parte Dunki*, 153 USPQ 678 (Bd.App. 1967) and *Clinical Products, Ltd.* v. *Brenner*, 255 F. Supp. 131, 149 USPQ 475 (D.D.C. 1966).

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-4 are rejected under 35 U.S.C. 102(b) as being anticipated by Brugidou C. et al. (The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. Mol Gen Genet. 1993 Apr;238(1-2):285-93).

The claims are drawn to a promoter comprising a nucleic acid fragment comprising at least one specific functional domain of the promoter of the TaTrxh2 gene, including a fragment wherein the sequence extends from position -1 to position -1111 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -1 to position -83 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -451 to position -591 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -591 to position -1111 relative to the ATG codon of the TaTrxh2gene; a fragment wherein the sequence extends from position -228 to position -451 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -451 to position -591 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -83 to position -228 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence is that of the first intron of the TaTrxh2 gene. The claims are also drawn to an expression cassette and a recombinant vector.

Brugidou C. et al. teach a promoter comprising a nucleic acid fragment comprising at least one specific functional domain of the promoter of the *Nicotiana tabacum* thioredoxin h gene,

including fragments relative to the ATG codon and a first intron (page 288 Figure 2). Brugidou C. et al. also teach an expression cassette and a recombinant vector (page 286 column 2; page 288 Figure 2). The promoter taught by Brugidou C. et al. comprises at least one functional domain of the disclosed TaTrxh2 gene promoter (GC box, A sequence rich in adenine; see page 10 of the specification). Additionally, while Brugidou C. do not explicitly teach that their promoter is "of the TaTrxh2 gene", Brugidou C. need not teach this limitation in order to anticipate the rejected claims, as a name imposes no specific structural limitations on the claimed promoter sequences.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 5-7 and 9are rejected under 35 U.S.C. 103(a) as being unpatentable over Brugidou C. et al. (The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. Mol Gen Genet. 1993 Apr;238(1-2):285-93) in view of Cornejo M.J. et al. (Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol. 1993 Nov;23(3):567-81).

The claims are drawn to a promoter comprising a nucleic acid fragment comprising at least one specific functional domain of the promoter of the TaTrxh2 gene, including a fragment wherein the sequence extends from position -1 to position -1111 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -1 to position -83 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -451 to position

-591 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -591 to position -1111 relative to the ATG codon of the TaTrxh2gene; a fragment wherein the sequence extends from position -228 to position -451 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -451 to position -591 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence extends from position -83 to position -228 relative to the ATG codon of the TaTrxh2 gene; a fragment wherein the sequence is that of the first intron of the TaTrxh2 gene. The claims are also drawn to an expression cassette, a recombinant vector, a transformed plant cell, a transgenic plant including a monocotyledonous plant, and a method for controlling the expression of a gene of interest in a plant cell.

The teachings of Brugidou C. et al. are set forth above. Brugidou C. et al. also teach that expression of the *Nicotiana tabacum* thioredoxin h gene is detectable in all young tissues that contain dividing cells (page 290 column 2 and Fig. 6A).

Brugidou C. et al. do not teach a transformed plant cell, a transgenic plant including a monocotyledonous plant, or a method for controlling the expression of a gene of interest in a plant cell.

Cornejo M.J. et al. teach the use of the maize ubiquitin 1 promoter, first exon and first intron (UBI) for rice (*Oryza sativa* L. cv. Taipei 309) transformation experiments, and its expression in transgenic calli and plants (abstract). Cornejo M.J. et al. also identified stable transformants obtained from callus-derived protoplasts co-transfected with two chimeric genes, the genes consisting of UBI fused to the coding regions of the uidA and bar marker genes (UBI:GUS and UBI:BAR) (page 575 Figure 5). Cornejo M.J. et al. additionally teach that histochemical

localization of GUS activity revealed that UBI was most active in rapidly dividing cells (abstract; page 576 Figure 6).

Given the teachings of Brugidou C. et al. that expression of the *Nicotiana tabacum* thioredoxin h gene is detectable in all young tissues that contain dividing cells, and given the teachings of Cornejo M.J. et al. that a promoter sequence obtained from a gene expressed in dividing cells can be further characterized by using that sequence to control the expression of a reporter gene of interest in a plant cell or plant transformed therewith, it would have been *prima facie* obvious to one skilled in the art at the time the invention was made to further characterize the *Nicotiana tabacum* thioredoxin h gene promoter sequence taught by Brugidou C. et al. by using that sequence to control the expression of a reporter gene of interest in a plant cell or plant transformed therewith as taught by Cornejo M.J. et al. One skilled in the art would have been motivated to do so in order to confirm the specific functional characteristics of the *Nicotiana tabacum* thioredoxin h gene promoter sequence. One skilled in the art would have had a reasonable expectation of success in view of the identification of specific functional motifs in the *Nicotiana tabacum* thioredoxin h gene promoter sequence, and in view of the general state of the art with respect to the functional characterization of promoter sequences as exemplified by Cornejo M.J. et al. Accordingly, one skilled in the art would have been motivated to generate the claimed invention with a reasonable expectation of success. Thus, the claimed invention would have been *prima facie* obvious as a whole to one of ordinary skill in the art at the time the invention was made.

Remarks

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Cynthia Collins whose telephone number is (571) 272-0794. The examiner can normally be reached on Monday-Friday 8:45 AM -5:15 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Jones can be reached on (571) 272-0745. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Cynthia Collins
Primary Examiner
Art Unit 1638

CC

Cynthia Collins
9/1/05

Notice of References Cited	Application/Control No. 09/979,549	Applicant(s)/Patent Under Reexamination GAUTIER ET AL.	
	Examiner Cynthia Collins	Art Unit 1638	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Brugidou C. et al. The Nicotiana tabacum genome encodes two cytoplasmic thioredoxin genes which are differently expressed. Mol Gen Genet. 1993 Apr;238(1-2):285-93.
	V	Cornejo M.J. et al. Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol. 1993 Nov;23(3):567-81.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

09/979549

JC19 Rec'd PCT/PTO 20 NOV 2001
19

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. ORES10.001APC	APPLICATION NO. Unknown
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICANT Gautier, et al.	
(USE SEVERAL SHEETS IF NECESSARY)	FILING DATE Herewith	GROUP Unknown

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE (IF APPROPRIATE)

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
CC	1.	WO 9603505	2/8/96	WO			X (Abstract)	

EXAMINER INITIAL		OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
CC	2.	Brugidou, et al. (1993) The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. Mol. Gen. Genet. 238:285-293

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EXAMINER Cynthia Collins	DATE CONSIDERED 8/31/05

*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED, INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

DUPLICATE OF IDS
Received 19 NOV 2001

JC19 Rec'd PCT/PTO 30 MAY 2002

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. ORES10.001APC	APPLICATION NO. Unknown 09/979549
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICANT Gautier, et al.	
(USE SEVERAL SHEETS IF NECESSARY)	FILING DATE Herewith	GROUP Unknown

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE (IF APPROPRIATE)

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
CC	1.	WO 9603505	2/8/96	WO			X (Abstract)	

EXAMINER INITIAL		OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
CC	2.	Brugidou, et al. (1993) The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. Mol. Gen. Genet. 238:285-293

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EXAMINER Cynthia Collins	DATE CONSIDERED 8/31/05

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Plant Molecular Biology 23: 567–581, 1993.

Activity of a maize ubiquitin promoter in transgenic rice

Maria-Jesús Cornejo[1]*, Diane Luth[2], Kathleen M. Blankenship[3], Olin D. Anderson and Ann E. Blechl

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Received 9 April 1993; accepted in revised form 28 July 1993

Key words: GUS expression, heat shock, *Oryza sativa* L., rice transformation, ubiquitin

Abstract

We have used the maize ubiquitin 1 promoter, first exon and first intron (UBI) for rice (*Oryza sativa* L. cv. Taipei 309) transformation experiments and studied its expression in transgenic calli and plants. UBI directed significantly higher levels of transient gene expression than other promoter/intron combinations used for rice transformation. We exploited these high levels of expression to identify stable transformants obtained from callus-derived protoplasts co-transfected with two chimeric genes. The genes consisted of UBI fused to the coding regions of the *uidA* and *bar* marker genes (UBI:GUS and UBI:BAR). UBI:GUS expression increased in response to thermal stress in both transfected protoplasts and transgenic rice calli. Histochemical localization of GUS activity revealed that UBI was most active in rapidly dividing cells. This promoter is expressed in many, but not all, rice tissues and undergoes important changes in activity during the development of transgenic rice plants.

Introduction

Ubiquitin is an eucaryotic protein consisting of 76 highly conserved amino acid residues and found in most cell types either as free monomers or conjugated to a variety of cytoplasmic, nuclear or membrane proteins (for a review see Hough *et al.* [30]). Ubiquitin has been implicated in multiple cellular functions, including protein degradation [28, 40] and response to heat shock (HS) and other stresses [7, 21, 10, 15, 5, 23].

Ubiquitin is encoded by multigene families of two general types: genes encoding tandem repeats of ubiquitin monomers (polyubiquitins) and genes encoding ubiquitin fusions with other proteins (ubiquitin fusion proteins). Among higher plants, members of these two classes of gene families have been characterized at the molecular level [24, 10, 13, 5, 6, 29, 16, 25, 41]. Overall, these studies show the tissue specificity of different transcript size classes and the regulation by environmental stresses of some ubiquitin genes and mRNA size classes. The only plant ubiquitin genes whose expression has been characterized at the cellular level are the ubiquitin extension protein genes *UBQ 1* and *UBQ 6* from *Arabidopsis*

thaliana [13]. Callis *et al.* [13] have shown that promoters from these two genes are expressed in all organs of transgenic tobacco. No comparable data have been reported thus far for polyubiquitin gene promoters or for any monocot ubiquitin genes.

Christensen *et al.* [16] have isolated and sequenced two polyubiquitin genes, *Ubi 1* and *Ubi 2*, from maize. Both genes contain an intron in their 5′-untranslated regions. Both are expressed constitutively at 25 °C in maize seedlings and are inducible to higher levels upon heat shock. Transient expression in maize protoplasts from the *Ubi 1* promoter, first exon and first intron (UBI) was more than 10-fold higher than expression from the cauliflower mosaic virus (CaMV) 35S promoter, using chloramphenicol acetyl transferase (CAT) as the reporter gene. The latter promoter has been widely used to drive marker gene expression in cereals, including both japonica and indica subspecies of rice [44, 27, 19, 47, 17, 20, 39].

The relative strength of the UBI promoter in maize protoplasts suggested that it might be suitable for driving high levels of gene expression in stable transformants of rice and other cereals. In this report, we demonstrate its usefulness for this purpose and characterize the expression pattern of a UBI:GUS fusion gene in transgenic rice plants. We also show that UBI is highly expressed in transfected protoplasts and transgenic calli of rice and that these levels of expression increase in response to HS.

Materials and methods

Isolation, transformation and culture of callus-derived protoplasts

We used mature-embryo-derived callus as a direct source of protoplasts for rice transformation, bypassing the time-consuming establishment of suspension cultures. To initiate callus lines, caryopses of rice (*Oryza sativa* L. cv. Taipei 309) were husked, sterilized for 45 min in 20% (v/v) commercial bleach and 0.1% (v/v) Tween 20, then cultured in a MS medium composed of MS salts [37], R-2 vitamins [38], 3% (w/v) sucrose and 0.7% (w/v) agarose, pH 5.7. This MS medium was supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction and maintenance. Calli were subcultured every three weeks.

Protoplasts were prepared by incubating small pieces of rice calli at room temperature with slow shaking in the following digestion mixture: 5 mM morpholinoethane sulfonic acid (MES), 5 mM $CaCl_2$, 0.55 M mannitol, 0.5% (w/v) bovine serum albumin (BSA), 2% (w/v) cellulase Onozuka R-10 and 1% (w/v) pectinase Macerozyme R-10 (both enzymes from Yakult Honsha, Tokyo, Japan)[1], and 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan). The pH of this digestion mixture was adjusted to 5.7 and 1 mM 2-mercaptoethanol was added before filter sterilizing.

After 2.5 to 3 h of incubation, protoplasts were sequentially filtered through nylon screens of 200, 50 and 30 μm mesh size and washed twice with 5 mM MES, 5 mM $CaCl_2$ and 0.5 M mannitol. Protoplasts were then resuspended in a mannitol-calcium buffer composed of 0.2 M mannitol and 80 mM $CaCl_2$, pH 5.7, counted and the concentration adjusted to 2–3 $\times 10^6$ protoplasts/ml. Protoplast viability was determined with methylene blue as described by Huang *et al.* [31].

Samples of the protoplast suspension (1 or 2 ml) were mixed first with 15 μg supercoiled plasmid DNA in 15 μl TE, pH 8 [43], then with equal volumes of 35% (w/v) polyethylene glycol 4000 (Sigma). The mixture was incubated at room temperature for 30 min. After transformation, protoplasts were washed twice and resuspended in simplified KPR medium [34]. The resuspended protoplasts were cultured following procedures described for cell-suspension-derived protoplasts by Abdullah *et al.* [1] as modified by Zhang and

[1] The use of a brand name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

Wu [53], except that protoplasts were placed on 0.45 μm Millipore filters (Millipore, Bedford, MA) over a layer of feeder nurse cells [14]. After 2 to 4 weeks, colonies were transferred from the filters to N_6 medium (N_6 salts [18], pH 5.8, 2% (w/v) sucrose, 100 mg/l casein hydrolysate, 2.9 g/l *L*-proline, 2 mg/l glycine, 1 mg/l thiamine-HCl, 0.5 mg/l each of nicotine acid and pyridoxine, 5 μM 2,4-D). Thereafter they were cultured individually at 27 °C in the dark.

After about 2 months, calli derived from protoplasts co-transfected with UBI:GUS and UBI:BAR were screened for β-glucuronidase (GUS) activity by histochemical staining [33]. Small pieces of calli were immersed in a solution of 3.3 mg/l 5-bromo-4-chloro-3-indolyl-β-*D*-glucuronide (X-Gluc), 0.1 M sodium phosphate buffer pH 7, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.25% (v/v) Triton X-100 and incubated in darkness. The blue precipitate that developed indicated the presence of enzyme activity. Calli showing high GUS activity were removed from the X-Gluc solution after 5 to 10 min, washed in liquid N_6 and then cultured on solidified N_6 medium.

Transformed and control (untransformed) callus lines were maintained in MS medium with 10 μM 2,4-D and kept in darkness at 27 °C. For plant regeneration calli were transferred to MS medium with 1 μM 2,4-D for 3 weeks, then transferred to MS medium with 1 μM 6-benzylaminopurine and incubated under a 16 h photoperiod for 3 to 6 weeks. Rooted plantlets were moved to pots and kept under mist sprayers in the greenhouse until flowering.

Plasmid constructs

Plasmid UBI:GUS, UBI:Luciferase and UBI:BAR consist of the maize *Ubi 1* promoter and 5′-untranslated sequence (including the first intron) [16] fused to the coding regions of the marker genes followed by the 3′-untranslated region and polyadenylation signal of the nopaline synthase gene (Nos 3′) [4]. UBI:Luciferase is the name used in this paper for pAHC18 [9]. In UBI:GUS, the GUS marker gene is substituted for luciferase. Both are similar to 'UBI:CAT' [16] and were kindly provided by Alan Christensen and Peter Quail (Plant Gene Expression Center, UC-Berkeley/USDA, Albany, CA) (A.H. Christensen and P.H. Quail, in preparation). UBI:BAR was constructed by Douglas Gurian-Sherman (Western Regional Research Center, ARS, USDA, Albany, CA). It consists of *Ubi 1* nucleotides −893 to + 1099 (Fig. 2 of Christensen *et al.* [16]) fused to the BAR coding and Nos 3′ regions of pBARGUS [22] in vector pUC8 [52]. BAR is the sequence from the *bar* gene of *Streptomyces hygroscopicus* that encodes phosphinothricin acetyl transferase (PAT) [50]. This enzyme inactivates phosphinothricin, an inhibitor of glutamine synthetase and the toxic ingredient in the herbicides Bialaphos and BASTA. Schematic diagrams of UBI:GUS and UBI:BAR are shown in Fig. 1.

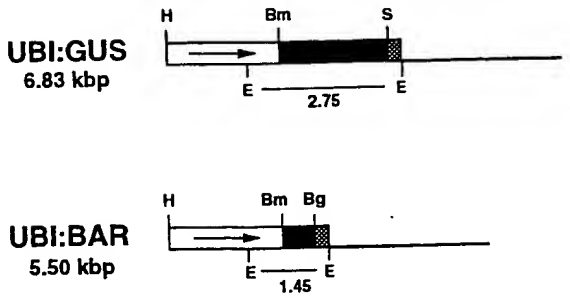

Fig. 1. Diagram of UBI:GUS and UBI:BAR plasmids. The relative sizes of various parts of the plasmids are drawn to scale. The solid rectangles are the coding regions for GUS and BAR used as probes in the DNA analyses in Figs. 4 and 5. UBI is represented by an open rectangle with an arrow indicating the direction of transcription. The transcription termination sequences derived from the nopaline synthase gene are indicated by stippled rectangles. pUC18 vector sequences are shown as lines. The locations of restrictions sites mentioned in this report are shown. H, *Hin*d III, Bm, *Bam* HI; Bg, *Bgl* II, E, *Eco* RI; S, *Sac* I. Sizes in kb of *Eco* RI fragments homologous to the probes used in the DNA analysis are indicated below each diagram.

For the promoter/intron comparisons performed in transient assays, the following plasmids were used. The maize *Adh1* promoter/intron 1 was tested with the luciferase reporter gene in the plasmid pAI_1LN [11] and with the GUS re-

porter as part of the dual vector pBARGUS [22]. The CaMV 35S promoter/*Adh1* intron 1 was tested with the luciferase reporter as part of the dual vector pALSLUC [22] and with the GUS reporter in the plasmid BP1, which consists of the same promoter/intron and Nos 3′ sequences flanking the GUS-coding sequence in vector pUC8 (M. Fromm, personal comunication). All these plasmids were constructed by Michael Fromm (Monsanto Company, St. Louis, MO) and provided by Rosalind Williams (Plant Gene Expression Center, UC-Berkeley/USDA, Albany, CA). The rice actin 1 promoter/intron 1 was tested in the plasmid Act-1D-Gus [36], kindly provided by Ray Wu (Cornell University, Ithaca, NY).

Enzyme assays

For transient assays, protein extracts were prepared from protoplasts collected by centrifugation 30 to 40 h after transformation. Protoplasts were resuspended in the appropriate extraction buffers (see below) and sonicated briefly. Calli and leaf extracts were prepared by first grinding the tissue (ca. 50 mg fresh weight) in extraction buffers and then sonicating. Extracts were clarified by centrifugation for 5 min at 15000 × *g*. For HS experiments with transgenic calli, extracts were prepared at the end of the incubation periods, stored at −70 °C and analyzed all together at a later time. Total protein concentrations in extracts were determined by the Bradford method [8] with a BioRad Laboratories kit (Richmond, CA) and BSA as the standard.

For transient luciferase and GUS assays, cells were extracted in 400 μl of a buffer composed of 100 mM potassium phosphate, pH 7.8, 1 mM disodium EDTA, 7 mM 2-mercaptoethanol and 0.5 mg/ml BSA.

To determine luciferase activity, 20 μl of protoplast extracts were mixed in cuvettes with 200 μl of assay buffer (25 mM Tricine, 15 mM $MgCl_2$, 5 mM ATP, 7 mM 2-mercaptoethanol and 0.5 mg/ml BSA). After 30 min, the cuvettes were placed in the luminometer (Monolight 2001, Analytical Luminescence Laboratories, San Diego, CA), 100 μl of 0.5 mM luciferin (K^+ salt, Analytical Luminescence Laboratories) added, and the number of light units in 10 s recorded.

GUS activity was quantitated in 150 μl of protoplast extracts by the fluorimetric assay [33]. The substrate, 2 mM 4-methyl umbelliferyl-β-*D*-glucuronide (MUG) was purchased from Molecular Probes, Eugene, OR. Methyl umbelliferone (MU, Sigma) levels in the reaction mixture were calculated from fluorescence measurements in a TKO 100 DNA Mini-Fluorometer (Hoefer Scientific, San Francisco, CA). Specific GUS activity was expressed as pmol MU/min per μg of protein.

PAT activity was assayed as described by Spencer *et al.* [46]. ^{14}C-labelled products were visualized by autoradiography (Kodak XAR-5 film) overnight. The incorporation of ^{14}C into the acetylated phosphinothricin products was quantified by computing the integrated optical density of the bands with whole-band analysis software (Bio Image, Ann Arbor, MI).

Herbicide application

Resistance to phosphinothricin (active ingredient of Bialaphos and Basta) was examined in the R_0 generation plants. Rice leaves from untransformed and transformed plants were painted near the tips with a 1% (v/v) BASTA TX solution and 0.1% (v/v) Tween 20. Changes in the morphology and pigmentation of leaves were monitored for 4 to 6 days.

DNA gel blot analysis

Genomic DNA was isolated from callus and leaf tissue essentially as described by Gordon-Kamm *et al.* [26], except that the process was scaled down to samples of ca. 300 mg which could be ground with a pestle in 1.5 ml microfuge tubes containing ice-cold extraction buffer. DNA was digested with *Eco* RI as specified by the manufacturer except that 4 mM spermidine was included in the digestions that were incubated for 4 h.

Approximately 3 μg of digested and undigested genomic DNAs were electrophoresed through 0.8% agarose (FMC, Rockland, ME) in TAE buffer [43] and transferred [45] onto a Nytran membrane (Schleicher and Schuell, Keene, NH). DNA was fixed to the membrane by UV cross-linking (Stratagene, La Jolla, CA) and hybridized to DNA fragments labelled with ^{32}P-dCTP (Dupont NEN) using the Random Primed DNA labelling kit supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN). The probes consisted of the coding regions of the GUS and BAR genes, isolated as an 1870 bp *Hin*d III/*Sac* I fragment from plasmid pBI 101 (Clontech, Palo Alto, CA) and a 574 bp *Bam* HI/*Sac* I fragment from UBI:BAR, respectively (filled rectangles in Fig. 1). Both prehybridization and hybridization were carried out at 65 °C in 6 × SSC, 5 × Denhardt's solution, 20 mM Tris HCl pH 7.6, 0.8% SDS, 100 μg/ml denatured salmon sperm DNA. After overnight hybridization, membranes were briefly washed with 1 × SSPE, 0.2% SDS, then washed twice for 45 min each with 0.25% SSPE, 0.2% SDS. Damp filters were exposed at −70 °C on Kodak XAR-5 film with an intensifying screen (Dupont). For rehybridization, probes were removed from the blots by boiling in 0.1 × SSPE, 0.2% SDS for 30 min.

Histochemical localization of GUS activity

GUS activity was detected in small fragments of leaves and roots essentially as detailed above for rice callus, except that the tissues were incubated at 37 °C. To stop the reaction, tissues were washed twice in 50 mM sodium phosphate buffer (pH 7). Leaf and root fragments were then fixed in 0.05 M sodium phosphate, 0.5 M sucrose and 4% paraformaldehyde for 2½ h followed by three washes (10 min each) in 50 mM sodium phosphate buffer and 0.5 M sucrose. Samples were dehydrated in ethanol dilutions (30%, 50%, 70%, 95%) for 20 min each and three times (20 min each) in 100% ethanol. Tissue samples were infiltrated and embedded using a LKB 2218-500 Historesin embedding kit (LKB, Bromma, Sweden). Samples were first infiltrated for 1 h with 50% resin and 50% absolute ethanol, left overnight in 100% resin and for 2 days with fresh resin and slow shaking. Samples were then embedded and left overnight at 4 °C to allow for slow and even polymerization. Samples were sectioned with glass knives and a Sorvall MT-2 (Dupont, Boston, MA) ultramicrotome. Roots were sliced in 2 μm sections and leaves in 4 to 10 μm sections. Tissue sections were viewed using a Zeiss Standard microscope with bright-field microscopy and photographed with a Nikon camera and Kodak Ektachrome 160 T film.

Results

A comparison of promoter strength

We used callus-derived protoplasts in transient assays to compare the activity of UBI to other promoter/intron combinations previously used for rice transformation. Transient assays were performed ca. 40 h after the introduction of DNA.

Figure 2 summarizes the results of five separate experiments, two for constructs containing the luciferase marker gene and three for GUS constructs. With either reporter gene, UBI directed transient gene expression at significantly higher levels than those obtained from CaMV 35S/*Adh* intron 1, rice actin/actin intron 1 and maize *Adh*/*Adh* intron 1. The luciferase-specific activity obtained from UBI (average value 2537 light units per μg protein) was 13-fold higher than the activity obtained from the 35S promoter and 6.8-fold higher than the activity from the *Adh* promoter. GUS-specific activity from UBI (average value 2 pmol MU per minute per μg protein) was 10.3-fold higher than the activity from the 35S promoter and more than 7-fold higher than the activities obtained from the *Adh* and actin promoters.

Effect of heat shock on transient GUS expression driven by the ubiquitin promoter

To study the effect of HS on transient gene expression, we incubated callus-derived protoplasts at 38 °C following transformation with

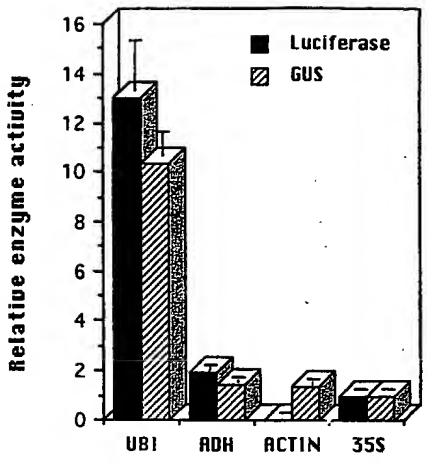

Fig. 2. Relative activity of several promoter/intron combinations in transfected rice protoplasts. Transient luciferase (solid bars) and GUS (striped bars) expression levels were determined 40 h after transformation. The following promoters/introns were used to drive transcription of reporter genes: UBI, maize *Adh1*/intron 1, rice actin 1/intron 1 and CaMV 35S/*Adh* intron 1. Relative enzyme activities are expressed as times the absolute values obtained with 35S/*Adh* intron 1 ± standard deviation of the ratios. This promoter/intron combination directed the lowest values of gene expression in all experiments: luciferase and GUS specific activities averaged 194.7 light units per μg protein and 0.12 pmol MU per minute per μg protein, respectively.

UBI:GUS. The experimental conditions influenced the response of the UBI:GUS gene in protoplasts. Table 1 shows that GUS activity varied with the duration of the HS treatment and the time elapsed between transfection and HS. Transient GUS expression decreased slightly when HS of 1 h and 6 h were applied only 30 min after transfection. HS of 12, 14 and 16 h, applied 6, 4 and 2 h after transfection respectively, increased GUS expression relative to that of control cells transfected with UBI:GUS, but incubated continuously at 27 °C for the total 18 h period.

To verify that the HS effect on GUS-specific activity was related to the regulation of the ubiquitin promoter by high temperature, we separated three sets of protoplasts from the same batch and transformed one set with 35S:*Adh* intron 1:GUS and the second with UBI:GUS. The third set was subjected to identical procedures but without adding any plasmid DNA. Samples of protoplasts from each set were incubated at 27 °C for 20 h or were allowed to recover at 27 °C for 3 h and then heat shocked for 10 h or 15 h. After HS, protoplasts were returned to 27 °C for the remainder of the 20 h incubation period.

Table 2 shows the effects of incubation under these treatments on protoplast viability and transient GUS expression. The number of living protoplasts counted 20 h after transfection decreased as a result of the HS treatment. Consistent with the decline in protoplast viability, GUS activity from the 35S promoter decreased 2-fold and 2.5-fold after HS treatments of 10 and 15 h, with respect to protoplasts transfected with the same construct but not subjected to HS. In contrast, the activity from UBI increased significantly with the duration of the HS over that of protoplasts not subjected to HS: 7.6-fold with 10 h HS and 7.9-fold with 15 h HS. Background GUS activity from the controls without added DNA decreased slightly with time of HS.

Table 1. Effect of the duration of heat shock (HS) and time of application on transient GUS activity.

Incubation conditions (18 h total)			GUS-specific activity (pmol MU per minute per μg protein)	X control (no DNA)
time (h) at 27 °C before HS	time (h) at 38 °C HS	time (h) at 27 °C after HS		
–	0	–	4.75	38.3
0.5	1	16.5	3.56	27.7
0.5	6	11.5	2.37	19.1
6	12	0	6.17	49.8
4	14	0	30	241.9
2	16	0	37	298.4

Table 2. Effects of heat shock on protoplast viability and transient GUS expression driven by the 35S and UBI promoters.

Heat shock time (h)	Average number of living protoplasts	GUS specific activity (pmol MU per minute per μg protein)		
		Control (no DNA)	35S[1] X control	UBI[1] X control
0	54×10^4	0.026	0.155[a] (5.9)	1.085[a] (41.7)
10	37×10^4	0.024	0.072[b] (3.0)	8.215[b] (342.3)
15	30×10^4	0.018	0.042[b] (2.3)	8.590[b] (477.2)

[1] Data were subjected to analysis of variance and means compared by Tukey's test. Within a column, values designated by the same letter are not significantly different at $P < 0.05$.

Selection of transformants

The high levels of transient gene expression driven by UBI suggested that it would be useful in identifying or selecting stably transformed rice tissues. To obtain rice transformants, we co-incubated protoplasts prepared directly from callus with plasmid DNAs UBI:GUS and UBI:BAR (Fig. 1), in the presence of PEG. To verify DNA uptake, aliquots of protoplasts were assayed for transient GUS activity. The average activity obtained from three independent protoplast transfections was 1.24 pmol MU per minute per μg protein (90.8 times the control). Calli derived from these protoplasts were tested for stable incorporation of the plasmid DNA by histochemical screening for GUS activity as described in Materials and methods. Out of 1282 colonies tested, we detected eight putative transformants (0.62%).

The transformed calli exhibited detectable blue staining after 5 to 10 min, indicating that the gene was expressed at high levels in the callus tissue. When GUS activities in six of these lines were quantified by the fluorometric assay (Table 3), we found levels of GUS activity in transformed calli more than 10000 times higher than levels in untransformed callus tissue. Although the solution utilized for the histochemical GUS assay is toxic to the plant cells, the time required to detect UBI-:GUS expression was so short that calli could be 'rescued' by washing them with culture medium and placing them back on solid medium (Fig. 6A). Following staining, the initial cell divisions presumably took place in the internal cells, where the assay solution had not penetrated. The newly grown cells were assayed fluorimetrically and had GUS activities similar to the original calli (data not shown).

Seven calli with GUS activity were tested for co-expression of the BAR gene. Five transformed GUS lines also exhibited PAT activity, the enzyme encoded by the BAR gene (Fig. 3). The levels of PAT in callus extracts were quantified by scanning the autoradiogram and determining the interoptical density (IOD) of the band corresponding to the acetylated product (Fig. 3,

Table 3. GUS and PAT activities in transformed callus lines identified by the histochemical GUS assay.

Callus line	GUS specific activity (pmol MU per minute per μg protein)	PAT activity (IOD values)[1]
Control	0.0018	0.198
B-2	11.19	0.189
B-4	16.64	3.471
B-7	21.15	4.542
B-8	28.23	7.074
B-9	28.37	8.184
B-10	ND[2]	0.177
B-11	19.85	2.167

[1] Interoptical density (IOD) values were determined as detailed in Materials and methods.

[2] Not determined.

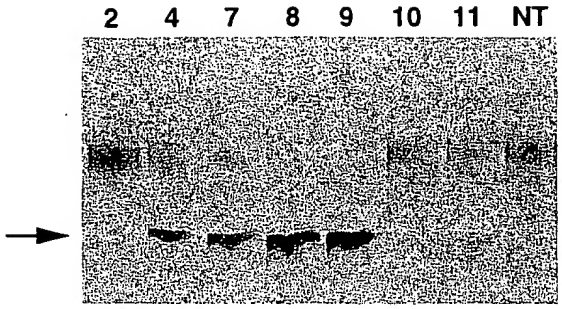

Fig. 3. PAT activity in transgenic rice callus lines. PAT activity in extracts from callus lines was determined as described in Materials and methods. Arrow indicates the migration position on the TLC plate of the acetylated form of phophinothricin, the product of the PAT enzyme activity. Transgenic callus lines called B-2, B-4, B-7, B-8, B-9, B-10 and B-11 in the text are indicated only by their number designations in this and subsequent figures. Lane NT contains extract from an untransformed callus line.

arrow). The quantified values are listed in Table 3 alongside the fluorometrically determined values for GUS activity in the same lines. Activity levels of the two enzymes are correlated ($r = 0.95$).

DNA analysis of transformants

DNA gel blot analysis was performed to confirm the integration of the UBI:GUS and/or UBI:BAR genes in the genome of seven calli exhibiting GUS and/or BAR activity. Figure 4 shows the genomic blot of undigested and *Eco* RI-digested DNA from these callus lines hybridized to the coding regions of either the UBI-:GUS or the UBI:BAR genes. The presence of sequences in the undigested DNA that hybridize to both the GUS and BAR coding regions suggests that the plasmid DNA had been integrated into the rice genome in these calli. When digested with *Eco* RI, the majority of the transformants showed multiple (7 to 14) bands homologous to the GUS probe (Fig. 4A). (A longer exposure of the lanes containing sample B-8 was needed to visualize the bands because these lanes contained less total DNA than the other lanes.) A 2.8 kb band (arrow), the size expected for unrearranged plasmid DNA (Fig. 1), was one of the most

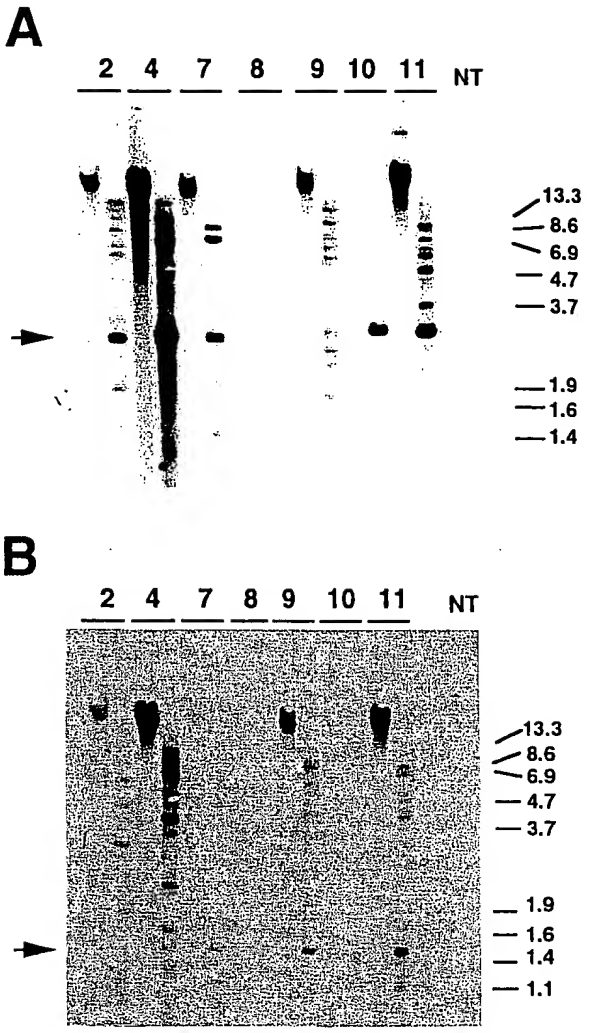

Fig. 4. DNA gel blot analysis of transgenic callus lines. For each numbered pair of lanes, undigested DNA is to the left and *Eco* RI-digested DNA is to the right. An arrow shows the predicted position of bands for the *Eco* RI fragments expected for unrearranged vectors. The NT lane contains *Eco* RI-digested DNA from an untransformed callus line. The sizes of molecular weight markers run on the same gel are indicated in kb to the right. For panel A, the probe was the GUS-coding region and for panel B, the same blot was probed with the BAR-coding region after removal of the GUS probe. The locations of the regions of the transforming plasmids homologous to the probes are shown as solid rectangles in Fig. 1.

prominent in each sample. Most additional bands were higher in molecular weight, indicating the loss of one or more *Eco* RI sites from the integrated vector DNA.

The GUS probe was removed and the blot was

re-hybridized to the BAR coding region (Fig. 4B). All of the transformant samples contained DNA homologous to the unselected co-transformed UBI:BAR plasmid DNA. Samples B-2 and B-10 did not have the 1.4 kb *Eco* RI band (arrow) expected for an intact BAR-coding region. These results are consistent with the enzyme data as these GUS transformants did not have any PAT activity. Each transformant that had the 1.4 kb band also expressed PAT activity.

Neither the GUS nor the PAT enzyme levels appeared to be correlated with the overall copy number or the number of intact copies of their respective transgenes. For example, transformant B-4 had the highest numbers of both transgenes but the second lowest enzyme activities (Table 3), while B-9 was among the lowest in GUS transgene copy number and yet had the highest level of enzymatic activity.

Effect of heat shock on gene expression in rice cells stably transformed with UBI:GUS

The effect of HS on the activity of the ubiquitin promoter was further tested in the transformed callus line B-2. Table 4 shows the levels of GUS activity in calli incubated at 38 °C for 12 h and subsequently at 27 °C, as compared to those incubated continuously at 27 °C. The response of UBI to HS in stable transformants was less pronounced (three-fold lower) than that of protoplasts, perhaps because of the high pre-existing levels of GUS protein in this cell line. The highest increase in GUS expression was 2.6-fold over controls (unheated calli from the same B-2 line), and occurred 2 days after the 12 h HS. The duration of the HS did not affect the kinetics of the ubiquitin response. Thus, after 2 h HS, the

Table 4. Effects of heat shock (HS of 38 °C for 12 h) and subsequent incubation at 27 °C on GUS activity in rice callus stably transformed with UBI:GUS.

Time after HS	GUS-specific activity[1] (pmol MU per minute per μg protein)
Control (no HS)	53.4[a, b]
2 hours	58.81[a, b, c]
5 hours	60.08[a, b, c]
1 day	101.20[c, d]
2 days	140.74[d]
3 days	90.60[b, c]
7 days	39.76[a]

[1] Data were subject to analysis of variance and Tukey's test. Values designated by the same letter are not significantly different at $P<0.05$.

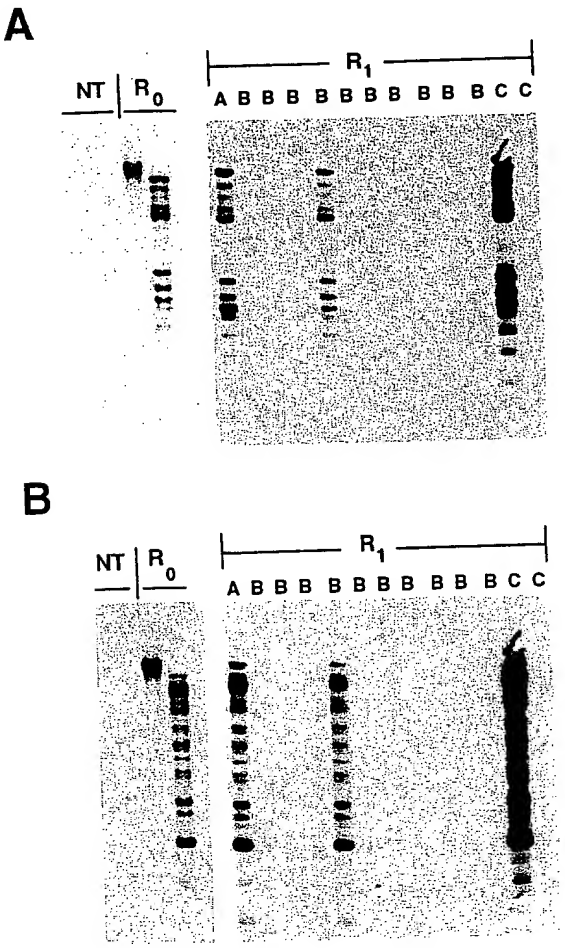

Fig. 5. DNA gel blot analysis of R_0 and R_1 transgenic plants. The NT pair of lanes contains undigested (left) and *Eco* RI-digested (right) DNA from a plant regenerated from untransformed callus. The R_0 pair of lanes contain undigested (left) and *Eco* RI-digested (right) DNA from a plant regenerated from the transgenic callus line B-9. The R_1 lanes contain *Eco* RI-digested DNAs from progeny of three different R_0 plants, A, B and C, regenerated from callus line B-9. For panel A, the probe was the GUS-coding region and for panel B, the same blot was hybridized to the BAR-coding region after removing the GUS probe.

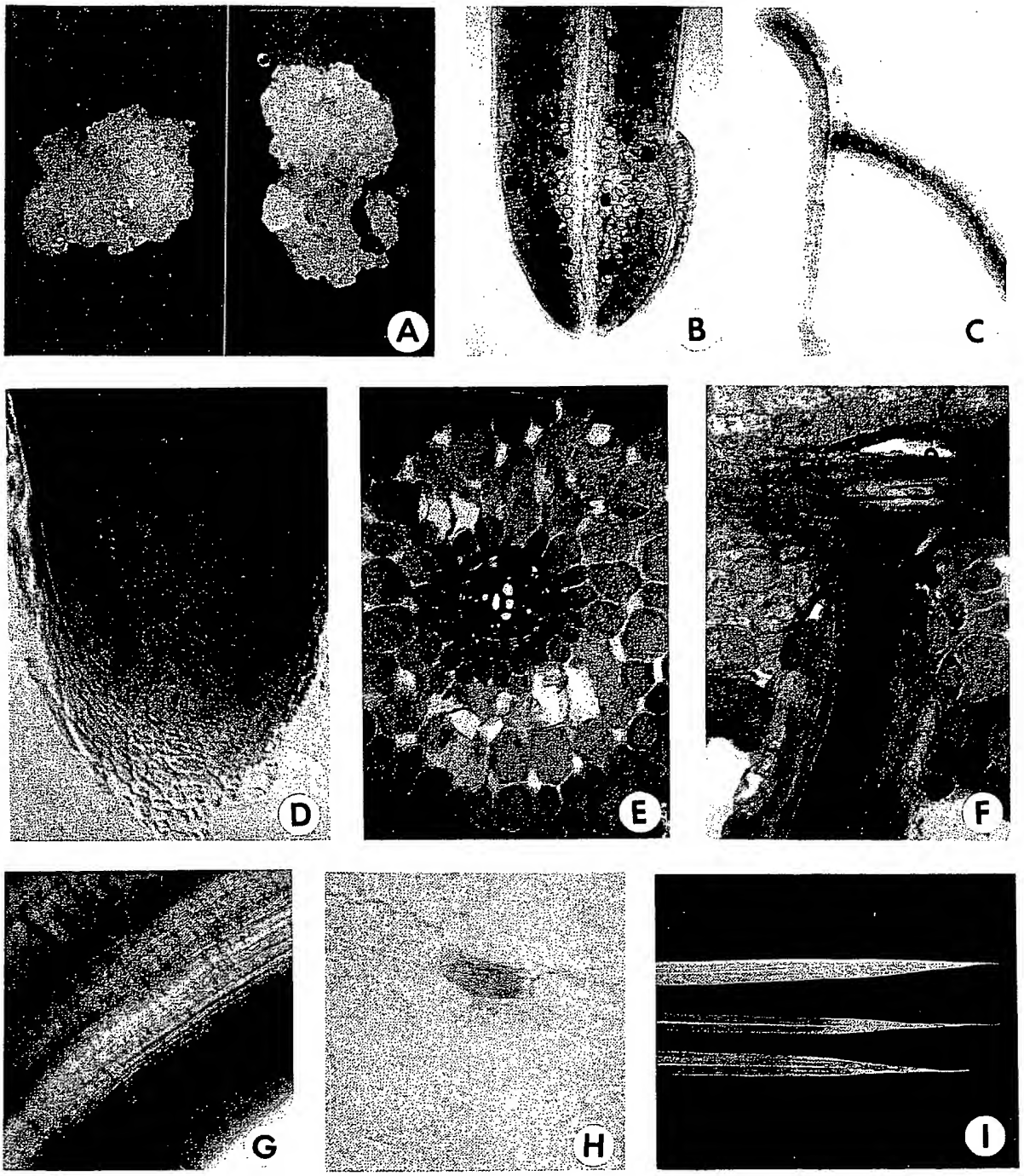

Fig. 6. Expression of the ubiquitin promoter in various tissues of transgenic rice. A. One week after screening with the histochemical GUS assay, cell proliferation continues in a transgenic callus (left). Three weeks later, this callus is growing actively (right). Only small blue areas remain from the original tissue exposed to the assay solution. B. Rice anther with a number of immature pollen

highest level of GUS expression (3.2-fold over controls) was also obtained after 2 days (data not shown). Seven days after either HS treatment, the levels of GUS activity had decreased and were comparable to those of controls.

DNA analysis of R_0 and R_1 plants

Plants were regenerated from the transformed callus line B-9, which co-expressed high levels of both GUS and PAT. Southern blot analysis of leaf genomic DNA from one of these R_0 plants is shown in Fig. 5. Undigested leaf genomic DNA from the transgenic plant contained a high molecular weight band at the position of chromosomal DNA that hybridized to both the GUS and BAR probes. The patterns of bands obtained with genomic DNA from the transgenic plants, cut with *Eco* RI, were similar to those of the progenitor callus line B-9.

Seed set in the transgenic R_0 plants was somewhat reduced compared to untransformed plants regenerated from tissue culture. As shown in Fig. 5, the transgenes in the B-9 plants were inherited in several of the R_1 progeny. Individual progeny contained either all the copies of the fragments homologous to the GUS- and BAR-coding regions or none of them, indicating that the inserted DNA sequences were inherited as a single genetic locus.

Expression of the ubiquitin promoter in various tissues of transgenic rice plants

Figure 6 shows the pattern of GUS expression driven by the ubiquitin promoter in R_0 plants as determined by the histochemical assay. A fraction of the pollen population obtained from R_0 plants (Fig. 6B) showed GUS activity, while the somatic tissue of the anthers remained unstained. The number of stained pollen grains and the intensity of staining varied among anthers from different buds. This may be related to the various degrees of pollen development found among flower buds.

GUS activity was very high in young roots. They appeared intensely stained throughout the meristematic, elongation and differentiation zones (Fig. 6D, E, F). Epidermis and cortex showed GUS activity in all cell types. Within the central cylinder (Fig. 6E) pericycle and phloem were stained. Only the xylem of the differentiation zone appeared unstained, as expected since these cells die during the development of the vascular system. GUS activity was very strong in lateral roots (Fig. 6F) and was also observed in root hairs (not shown). GUS expression decreased greatly as the roots became older (Fig. 6C).

Staining of transgenic leaves required overnight incubation in the assay solution. The stomata (Fig. 6H) and the vascular system (Fig. 6G) exhibited GUS activity. Thin sections showed the staining limited to the guard cells and the bundle sheath (not shown) of the vascular system. The fluorometric assay of leaf extracts from the same transgenic plant gave a GUS-specific activity of 8.25 pmol per minute per μg protein, 611.8-fold greater than that of a control extract prepared from untransformed leaves. As observed in roots, GUS activity decreased greatly in leaves as the plants aged and, during flowering, was only detectable following incubation at 38 °C (data not shown).

The resistance of mature rice leaves to the her-

grains showing different degrees of GUS staining. C. Mature root fragment showing faint GUS staining in the vascular system and strong staining in the vascular system of the lateral root. D, E and F. Histochemical localization of GUS activity in a young root. D. Longitudinal section of the meristematic and the elongation zones showing GUS activity in all cell types. E. Cross section of the differentiation zone showing GUS staining in epidermis and cortex. Within the central cylinder, pericycle and phloem are stained, but the xylem is unstained. F. Development of a lateral root from the pericycle. GUS staining is evident in all tissues. G. Fragment of leaf with GUS activity associated with the vascular system. H. Guard cells showing intense GUS staining; the surrounding tissue remains unstained. I. Effect of Basta TX on mature leaves of flowering plants. The herbicide was applied near the leaf tips. The control (upper leaf) appeared completely bleached four days after Basta application. The transgenic leaves (middle and lower leaves) showed local bleaching of leaf tips.

bicide Basta TX was examined at the time of flowering (Fig. 6I). The application of a 1% aqueous solution of Basta TX to leaf tips completely bleached the control, untransformed rice leaves in four days. The same Basta TX application in transgenic leaves caused localized and slight bleaching of leaf tips.

Discussion

The ability to obtain fertile transgenic rice plants has led to significant progress in studies on the expression of structural and regulatory gene sequences in this important cereal crop. In this report, we have used transient gene expression and transgenic plants to characterize quantitative and qualitative aspects of the activity of maize promoter sequences that regulate expression of the *Ubi 1* gene.

The feature of the maize *Ubi 1* promoter that makes it useful for rice transformation is its ability to drive very high levels of expression of marker genes in the calli typically used for selection or screening of rice transformants. In transient assays, UBI supported the highest levels of expression of any of the promoter/intron combinations that had been previously used to select rice transformants. Transient GUS expression from UBI was respectively 10.3-fold and more than 7-fold higher than that obtained from the 35S promoter/*ADH* intron 1 and maize *ADH* or rice actin promoters/introns. The high activity of the ubiquitin promoter in rapidly dividing cells allowed a clear-cut identification of stable transformants. Colonies expressing GUS were detected within minutes by the histochemical assay. In contrast, staining of colonies containing the *uidA* gene driven by the actin 1 promoter, which has the most constitutive expression of all promoters tested in rice, requires overnight incubation [54].

Recently, Toki *et al.* [51] obtained transgenic rice plants by electroporation-mediated transformation of protoplasts with UBI fused to the coding sequence of a *bar* gene and selection for resistance to the herbicide Bialaphos. We also have identified several calli transformed with UBI:BAR based on their ability to grow on media containing relatively low doses (0.5 mg/ml) of Bialaphos (M.J. Cornejo *et al.*, in preparation). Thus, the high expression levels of the maize *Ubi 1* gene promoter allow identification of rice transformants with either selectable or screenable marker genes.

UBI can also be used to drive expression of co-transformed genes. Five of seven transformants identified by UBI:GUS expression also exhibited activity from the unselected UBI:BAR genes. The activity levels of the different transgenes were highly correlated, perhaps indicating that the insertion sites for the two plasmids were equally permissive for transgene expression. In the case of transformant B-9, segregation analysis showed that indeed the two plasmids had co-integrated at the same or closely linked sites.

The distribution of GUS activity in transgenic rice plants reveals several other features of UBI expression. UBI is expressed in many, but not all, cell types and undergoes important changes in activity during the development of transgenic rice plants. Young roots showed very strong GUS expression levels. However, in more mature roots, expression decreased drastically and appeared only associated with the central cylinder, where the vascular tissues were still differentiating. GUS activity in rice leaves was located in the vascular system, and also in the guard cells. At the time of flowering, GUS expression was almost undetectable in the leaves.

Rubinstein and Reid [42] have shown a similar decline with age in the overall levels of ubiquitinated protein using polyclonal antibodies specific to ubiquitinated protein and tissue prints of cotton hypocotyls. Levels were much higher in younger (upper) than in older (lower) hypocotyl segments. In the older segments, the primary antibody accumulated mainly in the vascular tissue. Our experiments and those of Rubinstein and Reid, performed with different methodology, suggest that during the differentiation of some tissues there are high levels of ubiquitin, but these decrease drastically with time. Activity of ubiquitin genes has also been associated with a reversal of the differentiation process. Thus, Jamet

et al. [32] have reported an enhancement in the transcripts of some ubiquitin genes in *Nicotiana sylvestris* protoplasts derived from mesophyll cells.

The UBI pattern of expression suggests that this promoter is most active in cells with high metabolic activity and differs from other promoter activities that have also been characterized by GUS fusions in transgenic rice. The CaMV 35S promoter expression is widespread, exhibiting activity in and around the vascular tissue [49] and in most cell types of roots and leaves [3]. Activity of the maize *Adh1* promoter is restricted to root and shoot meristems, endosperm and pollen [35] and it is strongly induced in roots by anaerobiosis [53, 35]. Expression of the rice light harvesting chlorophyll a/b binding protein of photosystem II (LHCP II) has been detected in rice leaves, stems and floral organs, but not in roots [48]. The 5′ region of the rice actin 1 gene [54] is active in both vegetative and reproductive tissues of transgenic rice plants. Thus, its pattern of expression is more constitutive than those of CaMV 35S, maize *Adh1*, rice LHCP II and maize UBI.

In maize seedlings, the *Ubi 1* gene is expressed constitutively at 25 °C, but induced to higher levels at elevated temperatures [16]. Our data show that the maize *Ubi 1* promoter also responds to HS when introduced into an heterologous system. Transient expression of the UBI:GUS gene increased 7.9-fold in rice protoplasts that had been incubated at 38 °C for 15 h if they were allowed to recover for 3 h at 27 °C after transfection. However, when we incubated protoplasts at high temperature only 30 min after transformation, the GUS gene was expressed at a lower level than that obtained from unheated protoplasts.

Christensen *et al.* [16] did not find any heat-induced increase in CAT activity in maize protoplasts transformed with UBI:CAT. Similarly, Binet *et al.* [5], using the promoter region of two heat-inducible sunflower polyubiquitin genes and GUS as reporter gene, did not obtain any enhancement in expression in tobacco protoplasts transformed with these constructs and incubated at high temperature. In both cases, heat treatments were applied immediately after transformation. It seems, then, that protoplasts undergoing heat-shock shortly after DNA introduction have not had sufficient time to recover from the stress resulting from the isolation and transformation procedures. On the other hand, in maize protoplasts [12] and tobacco protoplasts [2] transformed with chimeric genes including heat-inducible promoters from the maize *hsp70* and soybean *Gmhsp*17.5-*E* genes, respectively, expression of reporter genes increased when heat treatments were applied 12 h (maize protoplasts) and 22 h (tobacco protoplasts) after transformation. Thus, in these systems and in rice, the length of the time between transformation and heat application seems to be critical in the induction of the HS response.

Transformed callus lines also exhibited increased levels of GUS expression following incubation at an elevated temperature. The 2.6-fold increase was of similar magnitude as that seen by Christensen *et al.* [16] in steady-state levels of the native *Ubi 1* transcript in maize cells three hours after a 10 min HS of 45 °C. Thus, UBI:GUS responded to HS whether integrated into the rice genome or as part of non-integrated plasmid DNA in the transient assay.

The activity of UBI in transgenic rice is consistent with the presence of ubiquitin in different cell types and with its proposed role in response to environmental stresses. Analyses of gene expression in transgenic plants are required to characterize the roles of other members of the ubiquitin multigene family during plant development.

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